Mitochondrial import: **Crossing the aqueous intermembrane space**

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Mitochondrial protein import follows a general pathway for preproteins with amino-terminal presequences. The discovery of novel import components has now revealed a distinct pathway for translocation of hydrophobic proteins across the intermembrane space and into the inner membrane.

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The vast majority of mitochondrial proteins are synthesized as preproteins on cytosolic polysomes and imported into the organelle. In the past ten years, the mechanisms and components of a general mitochondrial import pathway have been elucidated [1–4]. This general pathway mainly handles hydrophilic preproteins with amino-terminal presequences that target them to the mitochondria. Now, the discovery of a number of novel components of the mitochondrial import machinery has revealed a distinct, more specialized pathway that facilitates the translocation of hydrophobic proteins through the aqueous environment of the intermembrane space and into the inner membrane.

Preproteins with amino-terminal presequences

A typical mitochondrial preprotein contains an amino-terminal targeting sequence, termed the presequence. The presequence has the ability to form an amphipathic structure with a positively charged side and a hydrophobic side. The preprotein, probably escorted by cytosolic chaperones, is directed to the translocase of the outer mitochondrial membrane (Tom). The presequence is recognized by two receptors, Tom20 and Tom22, where the numbers indicate the approximate molecular masses of the components in kDa. With the help of the small protein Tom5, the preprotein is inserted into the general import pore (GIP) of the outer membrane (see Figure 1). At the *trans* side of the outer membrane, the presequence can interact with an intermembrane-space domain of Tom22, and is then transferred to the translocase of the inner membrane (Tim).

A Tim core complex, consisting of Tim23 and Tim17, forms a channel for the preprotein. First, the presequence is translocated across the membrane in a process that strictly depends on the membrane potential $\Delta\psi$ across the inner membrane (negative on the inside). Subsequently, on the matrix side of the inner membrane, the heat shock protein 70 (mtHsp70) binds to the unfolded polypeptide chain and promotes translocation of the mature part of the preprotein in an ATP-dependent manner. The chaperone mtHsp70 interacts transiently with the Tim machinery — Tim44 and the Tim core complex — and drives protein import by a combination of pulling and trapping of the polypeptide chain. In the matrix, a specific peptidase, the mitochondrial processing peptidase, removes the presequence. Assisted by further molecular chaperones, or unassisted, the imported protein folds to the active form.

On the tour across the mitochondrial membranes, the presequences successively interact with a number of negatively charged patches of Tom and Tim proteins: Tom20, Tom22 and Tom5 on the cytosolic side, as well as Tom22 and Tim23 on the intermembrane-space side. The negative potential inside the inner membrane further facilitates translocation. Stepwise interactions of presequences with negatively charged regions was suggested to be one mechanism by which preproteins are directed into mitochondria — the 'acid chain hypothesis'. It is evident, however, that additional forces besides ionic interactions are involved in protein import, and that the reaction cycle of mtHsp70 is a major driving force.

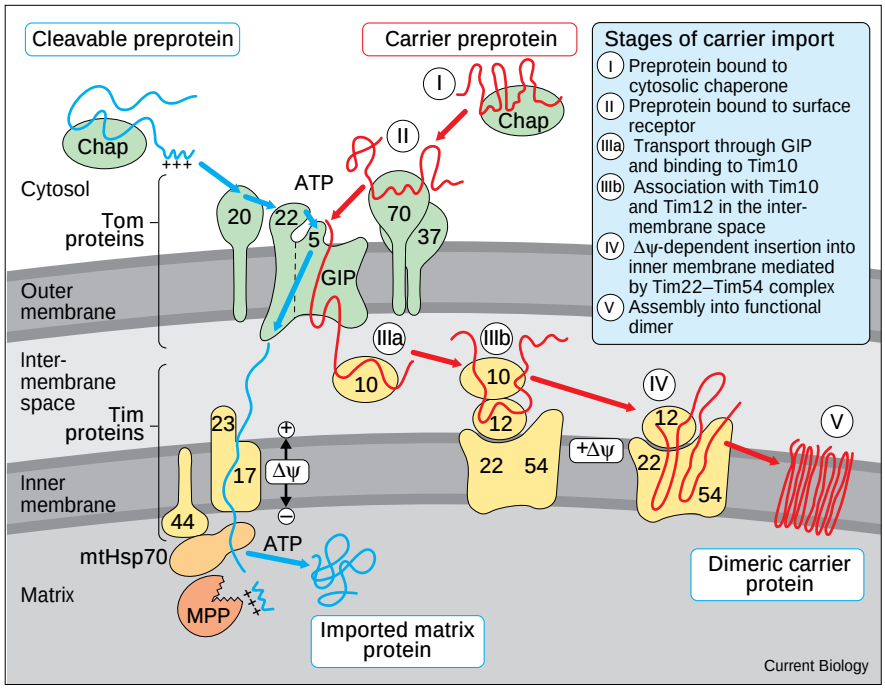
Variations on the general import pathway

A number of variations on this general import scheme have been described, particularly for proteins sorted to other mitochondrial subcompartments than the matrix [1–3]. Outer membrane proteins, which do not contain cleavable presequences, can use the general pathway via a receptor and the GIP, and are then released laterally into the outer membrane. This involves the action of a further small Tom protein, Tom7, which mediates dissociation of the Tom machinery. Some proteins of the intermembrane space, which are synthesized without a presequence, just use the Tom machinery to cross the outer membrane and then stay in the intermembrane space.

Other intermembrane-space proteins, and some inner membrane proteins, however, are made with presequences, and engage with the Tom and Tim machineries so that the presequence enters the matrix and is cleaved off by the processing peptidase. The exact sorting pathway of these proteins has not yet been determined and might vary depending on the preprotein studied, but current evidence indicates that their import pathway diverges from the general import pathway at the level of the inner membrane translocase.

These are, however, relatively small variations on the general pathway — the preproteins still use the typical components of the general import pathway, they just leave the pathway at earlier stages. The driving forces for preproteins that use only the outer membrane machinery are

Figure 1



Two pathways for import of preproteins into mitochondria. On the left is the pathway for import of proteins made as precursors with amino-terminal presequences. Such a protein is bound by cytosolic chaperones (Chap), interacts with the Tom20 and Tom22 receptors and is inserted into the GIP of the outer mitochondrial membrane with the help of Tom5. The preprotein interacts with the intermembrane space domain of Tom22 and is translocated across the inner membrane by the Tim23–Tim17 core complex, in a membrane potential (Δψ)-dependent manner. The mtHsp70 transiently interacts with Tim44 and the preprotein and drives the import in an ATP-dependent reaction. Mitochondrial processing peptidase (MPP) removes the presequence. On the right is the pathway for import of polytopic inner membrane proteins, for which metabolite carrier proteins are typical representatives. Such a protein preferentially uses the Tom70/Tom37 receptor and is directed into the GIP by Tom5 (Tom20 can also be used as the receptor, but with lower efficiency). At the *trans* side of the outer membrane, the import pathway for polytopic proteins diverges from that for cleavable preproteins. A carrier preprotein binds to Tim10, is transferred to Tim12, and is inserted into the inner membrane in a Δψ-dependent reaction at the Tim22–Tim54 complex; this is followed by release into the lipid phase of the membrane and dimerization to the functional form.

not clear. It is assumed that the energy gained from folding to the active forms in the outer membrane or intermembrane space drives the unidirectional translocation.

A new import pathway for integral inner membrane proteins

For a large class of integral inner membrane proteins, however, a pathway that is more radically different from the general one has now been discovered [5–8]. The pathway relies to only a small degree on the Tom and Tim proteins of the general import pathway, mainly using other Tom and Tim proteins (Figure 1). This pathway is used by polytopic membrane proteins — that is, proteins that span the inner membrane several times, including the large family of metabolite carriers and several Tim proteins. The metabolite carriers, such as the ADP/ATP carrier, the phosphate carrier and the dicarboxylate carrier, are homologous proteins and are functional as homodimers [9]. Each subunit consists of three related repeats of approximately 100 amino acids, implying that they are derived from the triplication of an ancient gene. Each repeat contains two membrane-spanning segments.

The exact targeting signals in these polytopic membrane proteins have not been identified so far. The targeting information is located within the mature protein part, and not at the extreme amino terminus. In the case of the ADP/ATP carrier, the presence of two independent targeting signals has been reported, one located in the amino-terminal third of the protein and the other in the carboxy-terminal two-thirds [10,11]. Functional studies indicate the presence of at least 20 different metabolite carriers in mitochondria, and the complete genome sequence of the yeast *Saccharomyces cerevisiae* has revealed the presence of 35 homologous genes for mitochondrial carrier proteins [9].

Although mitochondrial proteins made with presequences are mostly hydrophilic and contain hydrophobic segments in only a limited number of cases, the polytopic inner membrane proteins each contain from four to six hydrophobic segments. The hydrophobic segments strongly increase the probability that protein aggregation or misfolding will occur during transport through aqueous compartments, such as the cytosol and the intermembrane space. In the cytosol, molecular chaperones, such as Hsp70 or the mitochondrial import stimulation factor (MSF), bind to the preproteins and can shield the hydrophobic segments; this mechanism also facilitates the transport of hydrophobic outer membrane proteins [4]. A conceptual problem arises, however, from the observation that preproteins in transit from the outer to the inner membrane, including carrier proteins, are exposed to the intermembrane space [12,13]. There is no evidence that molecular chaperones of any of the known classes are present in the mitochondrial intermembrane space.

The answer relies on the recent identification of two new proteins, Tim10 and Tim12, located in the mitochondrial

intermembrane space. Both proteins can associate with the inner membrane, albeit in the case of Tim10 only very loosely. Tim10 and Tim12 were identified genetically — overexpression of their genes suppresses mitochondrial RNA splicing defects — and were originally termed Mrs11p and Mrs5p. Surprisingly, each of the genes *TIM10* and *TIM12* is essential for viability of the yeast *S. cerevisiae* under all growth conditions tested [14,15], although neither a mitochondrial splicing defect nor any defect in mitochondrial respiration is lethal on fermentable growth media. To date, lethal effects of deleting genes encoding mitochondrial proteins have virtually only been observed for components of the mitochondrial protein import and folding machinery [1–3].

Subsequent work showed that Tim10 and Tim12 are indeed essentials components of the protein import machinery specialized for translocating carrier proteins and other polytopic inner membrane proteins [7,8]. (The suppression of RNA splicing defects by *TIM10* and *TIM12* overexpression seems to be an indirect effect that may be explained by their products' function in mitochondrial protein import.) This work was complemented by the identification of two new inner membrane proteins, Tim22 and Tim54, which form a complex with which Tim12 and Tim10 can associate. Tim22 and Tim54 are also both essential for yeast cell viability, and are specialized for the carrier import pathway [5,6] (Figure 1). It is assumed that Tim10 and Tim12 perform chaperone-like functions by binding to the precursor chains of polytopic membrane proteins during the transfer through the aqueous intermembrane space, and associate with the Tim22–Tim54 complex where the $\Delta\psi$-dependent insertion into the inner membrane occurs.

From the characterization of the new Tim proteins [5–8], and earlier experimental dissection of the import pathway of the ADP/ATP carrier into distinct steps [16–18], we can now envisage a detailed picture of the import pathway of carrier proteins. Carrier protein precursors, escorted by cytosolic molecular chaperones (Figure 1, stage I), are directed to mitochondrial surface receptors, preferring the receptor Tom70 (Figure 1, stage II). Although Tom70 preferentially binds preproteins with internal targeting signals, and Tom20 and Tom22 prefer proteins with presequences, there is no strict separation of specificities. This means that parallel targeting pathways can be used, though with lower efficiency; on the one hand, carriers can interact with Tom20 (particularly when Tom70 has been removed) and then enter the GIP via the 'presequence pathway', and, on the other hand, some cleavable preproteins can bind to Tom70 prior to, or instead of, Tom20 [1–4]. In any case, the preproteins are transferred to the GIP complex and are inserted into the pore with the help of Tom5 [3], representing the short part of the import pathway that is common to both types of preprotein.

The import pathways separate as early as the *trans* side of the outer membrane. While presequence-containing preproteins can interact with the intermembrane-space domain of Tom22 [1,3], carrier proteins accumulate at the *trans* side independently of this domain [18]. Here Tim10 binds to the carrier proteins. In fact, Tim10 is needed to drive translocation of carriers across the outer membrane [7,8], indicating that it is functionally involved in formation of the *trans* binding site (Figure 1, stage IIIa). The carrier proteins then bind to Tim12, at an import stage where they have probably crossed the outer membrane (Figure 1, stage IIIb).

Tim10 and Tim12 do not show significant overall homology to other known proteins, but they are homologous to each other. Both proteins contain a zinc-finger-like motif with four cysteines and bind zinc ions [8]. The conformations of Tim10 and Tim12, and their interactions with carrier preproteins, are strongly influenced by metal ions, indicating that the zinc fingers are of structural or functional importance. Although zinc fingers are often found in transcription factors and are known as DNA-binding motifs, there is now evidence that they are present in numerous other proteins and may be involved in protein–protein interactions. Interestingly, the molecular chaperone DnaJ contains a zinc-finger-like domain that is involved in binding to denatured protein substrates, probably by stabilizing the polypeptide-binding region of the chaperone [19]. The presence of zinc fingers in Tim10 and Tim12 are thus compatible with their proposed chaperone-like function.

Tim10 and Tim12 transfer the carrier preproteins to the membrane-embedded Tim22–Tim54 complex, where the $\Delta\psi$-dependent insertion into the inner membrane occurs (Figure 1, stage IV). Tim22 was the first Tim component of the carrier pathway to be identified [5]. It shows homology to both Tim17 and Tim23, the integral proteins of the import pathway for cleavable preproteins, including conservation of the four membrane-spanning segments in each of the three Tims. Although carrier proteins can be inserted into the inner membrane independently of the Tim23–Tim17 core complex [3,5,20], basic mechanisms of the insertion process may be similar for both cleavable preproteins and carriers. The function of Tim54 is not yet clear; it associates with Tim22 and seems to be involved in the biogenesis and/or stabilization of Tim22 [6]. Finally, the carriers are released into the lipid phase of the inner membrane, where they form homodimers (Figure 1, stage V).

Two energy requirements for import of carrier proteins have been found so far. First, transfer of carriers from the receptor-bound stage (Figure 1, stage II) to the GIP-inserted stage (Figure 1, stage IIIa) requires nucleoside triphosphates, probably ATP [17]. The ATP-dependent step has not yet been directly pinned down; it may include the release from cytosolic chaperones [4] or the ATP-dependent action of a Tom protein. And second,

insertion into the inner membrane at the Tim22–Tim54 complex is strictly dependent on the membrane potential. How the membrane potential acts has not yet been resolved, but may include the induction of a conformational change in the Tim machinery, or an electrophoretic effect on positively charged segments of the carriers [1–3]. Additional driving forces for import may come from the energy freed by binding of carriers to import components, and the insertion and folding of carriers in the lipid phase of the inner membrane.

A link between the import machineries for cleavable preproteins and carriers?

Are the inner-membrane import machineries for cleavable preproteins and carrier proteins fully distinct entities? Recently, insertion of the ADP/ATP carrier into proteoliposomes was reconstituted using detergent-solubilized inner-membrane vesicles [21]. The insertion required a membrane potential and, surprisingly, Tim23. Although the efficiency of insertion was low, this result suggests that the Tim23–Tim17 complex may have a basic ability to mediate insertion of carrier proteins, or that the Tim23–Tim17 and Tim22–Tim54 complexes interact cooperatively.

It is interesting to compare our current knowledge of the Tim complexes with our developing understanding of the Tom complex. Most Tom proteins were identified before the first Tim proteins were discovered. The original picture of separate targeting pathways, still considered to represent the main import routes (Figure 1), has been complicated so that a network of receptor interactions is now invoked, conferred by the overlapping specificities and transient interactions of Tom subcomplexes [1–3]. Furthermore, in the case of the Tim machinery for cleavable preproteins, subcomplexes have been identified and two independent binding sites for mtHsp70 — on Tim44 and the Tim17–Tim23 complex — have been detected [22]. The few examples of cleavable preproteins that also contain several membrane-spanning segments may be examples of proteins that employ both Tim complexes.

Moreover, the role of mtHsp70 in the import of carrier proteins has not been finally clarified. While the import of carrier proteins, unlike that of cleavable preproteins, is not inhibited by depletion of matrix ATP [17,23], mutation of mtHsp70 does influence carrier import [24]. Future studies will have to investigate whether the mtHsp70 function of directly driving the translocation of cleavable preproteins into the matrix, which requires several ATP-dependent mtHsp70 reaction cycles, is complemented by an additional regulatory action of mtHsp70 on Tim complexes [20] that can operate at low levels of ATP.

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